BLOOD
The Journal of
The American Society of
Hematology

VOL 75, NO 9 CONTENTS MAY 1, 1990



Applicants: David J. Pinsky
U.S. Serial No.: 09/374,586
Filed: August 13, 1999
Group Art Unit: 1633

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# Analyses of Thrombocytopenia in Idiopathic Thrombocytopenic Purpura-Prone Mice by Platelet Transfer Experiments Between (NZW × BXSB)$F_1$ and Normal Mice

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**Male (NZW × BXSB) $F_1$ (W/B $F_1$) mice, which develop lupus nephritis, myocardial infarction, and thrombocytopenia, showed reduced platelet lifespan (PLS) and increased platelet-associated antibody (PAA) values. There were statistically significant correlations between the increase in PAA values and either the reduction in PLS or the decrease in platelet counts. This and the results of platelet transfer experiments between old male W/B $F_1$ mice and either female W/B $F_1$ or normal BALB/c mice indicate that PAAs on the platelet surface play a crucial role in the destruction of platelets in W/B $F_1$ mice. The mechanism of thrombocytopenia observed here appears similar to that of human idiopathic thrombocytopenic purpura (ITP). Therefore, we think that W/B $F_1$ mice are a potentially useful animal model for investigating the effectiveness and mode of action of therapeutic agents in human ITP, and that they may provide additional information on the basic mechanisms of this autoimmune phenomenon.**


IT IS THOUGHT that the autoimmune mechanism is involved in the development of chronic idiopathic (immune) thrombocytopenic purpura (ITP); the binding of circulating anti-platelet antibodies to platelets results in their destruction by phagocytic cells in the reticuloendothelial system.[1,2] The presence of anti-platelet antibodies, and a markedly reduced platelet lifespan (PLS), have been among the accepted clinical features of human ITP.[3,4]

We recently reported that male (NZW × BXSB)$F_1$ (W/B $F_1$) mice, which develop lupus nephritis with myocardial infarction, show thrombocytopenia with age, and that this is probably due to the presence of both platelet-associated antibodies (PAAs) and circulating anti-platelet antibodies.[5] In this study, we investigate the PLS of W/B $F_1$ mice and performed platelet transfer experiments between male W/B $F_1$ mice and either female W/B $F_1$ or normal BALB/c mice. We found that the platelets of male W/B $F_1$ mice show significantly reduced lifespans with age, and that there are statistically significant correlations between the increased PAA values and either the reduced PLS or the decreased platelet counts.

## MATERIALS AND METHODS

*Mice.* BALB/c, BXSB, and NZW mice were originally purchased from Jackson Laboratories (Bar Harbor, ME). W/B $F_1$ (hybrid of NZW female and BXSB male) were raised under specific pathogen-free conditions in the animal facility of the Nippon Shinyaku Research Laboratories (Kyoto, Japan).

*Platelet labeling and platelet kinetics studies.* $^{111}$In-tropolone was used for the labeling of platelets by the method of Dewanjee et al.[6] It was made by mixing 100 μL of $^{111}$In-0.04 mol/L HCl (Japan Medi-physics Co, Hyogo, Japan, 1 mCi/mL) and the same volume of tropolone solution (Sigma Co, St Louis, MO) with acid-citrate-dextrose (ACD-A, pH 4.5, 0.01 mol/L)-saline at a concentration of 2 mg/mL, then incubated for 5 minutes at room temperature. Blood (1.5 mL) was obtained from the retro-orbital plexus of ether-anesthetized mice in ACD-A. Platelets were isolated by differential centrifugation at room temperature, first at 300 × *g* for 5 minutes to obtain platelet-rich plasma, which was then washed three times at 2,000 × *g* for 15 minutes. Contaminating red blood cells (RBCs) were removed by centrifugation at 100 × *g* for 5 minutes. Platelets ($1 \times 10^9$) were resuspended in 1 mL of ACD-A-saline (pH 7.6), to which 100 μL of $^{111}$In-tropolone was added. The mixture was kept under constant agitation for 30 minutes at room temperature, and the radiolabeled platelets were then washed three times in ACD-A-saline and resuspended in 1.0 mL of saline. The labeling indices ranged from 50% to 70%. Recipient mice were injected intravenously (IV) with the radiolabeled platelets (3 to 5 μCi in 0.2 mL). Blood (15 μL) was taken 1 minute, 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours, and 48 hours after injection. The radioactivity was counted using a well-typed scintillation counter (Aloka Co, Tokyo, Japan).

*Data analysis.* The mean platelet survival was estimated by calculating the rate of platelet disappearance from circulation using multiple-hit model analyses, as recommended by the International Committee for Standardization in Hematology.[7]

*Platelet-associated antibodies.* Because of the difficulty of obtaining sufficient blood samples, we used a modified method of platelet suspension immunofluorescence test (PSIFT) for PAA.[5,8] Briefly, platelet suspensions were obtained from EDTA blood (50 μL) by centrifugation as mentioned above. After washing three times in 5 mmol/L EDTA-phosphate-buffered saline (PBS) (pH 6.8), the platelets were suspended in 1% paraformaldehyde solution for 5 minutes. Platelets were resuspended in 100 μL of EDTA-PBS to a platelet count of $3 \times 10^5$/μL. Then they were incubated with 5 μL of FITC-cojugated goat anti-mouse immunoglobulin (Ig) (Becton Dickinson, Sunnyvale, CA) for 30 minutes at room temperature. After final washing, samples were analyzed on a FACScan (Becton

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*Submitted May 23, 1989; accepted January 9, 1990.*
*Supported in part by grants from the Japanese Ministry of Health and Welfare; Naito Foundation; Mitsubishi Foundation; a Grant-in-aid from the Mochida Memorial Foundation for Medical and Pharmaceutical Research; a grant from Suzuken Memorial Foundation; the Science Research Promotion Fund of the Japan Private School Promotion Foundation; and a Grant-in-aid for cancer research, 01015110 (1989) and a Grant-in-aid for general scientific research, 63480147 (1988) from the Ministry of Education, Science, and Culture.*
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*0006-4971/90/7509-0015$3.00/0*

**Table 1. Platelet Lifespans, Platelet-Associated Antibodies, and Platelet Counts in W/B $F_1$ Mice**

| Mouse | Age (mo) | Sex | No. | Platelet Count ($\times 10^{-3}/\mu L$) | PAA* (%) | Platelet Lifespan† (d) |
|---|---|---|---|---|---|---|
| BALB/c | 6 | M | 6 | 1,225 ± 153 | 1.6 ± 1.0 | 2.11 ± 0.28 |
| NZW | 6 | M | 5 | 1,005 ± 113 | 1.6 ± 0.9 | 1.62 ± 0.61 |
| BXSB | 4 | F | 5 | 962 ± 91 | 1.9 ± 0.5 | 1.57 ± 0.33 |
| W/B $F_1$ | 3 | F | 3 | 1,012 ± 55 | 3.0 ± 2.4 | 1.44 ± 0.21 |
| W/B $F_1$ | 1 | M | 4 | 1,063 ± 114 | 1.4 ± 1.2 | 1.20 ± 0.40 |
| W/B $F_1$ | 2 | M | 4 | 1,021 ± 140 | 10.1 ± 6.1‡ | 0.56 ± 0.04‡ |
| W/B $F_1$ | 3 | M | 4 | 842 ± 57 | 18.9 ± 15.0‡ | 0.25 ± 0.11‡ |
| W/B $F_1$ | 4 | M | 4 | 612 ± 104‡ | 15.9 ± 5.8‡ | 0.09 ± 0.03‡ |
| W/B $F_1$ | 5 | M | 4 | 502 ± 93‡ | 25.5 ± 4.6‡ | 0.12 ± 0.03‡ |
| W/B $F_1$ | 6 | M | 4 | 491 ± 72‡ | 49.9 ± 5.2‡ | 0.11 ± 0.04‡ |

*Platelets were labeled with FITC-conjugated goat anti-mouse Ig and analyzed using a FACScan.

†Platelet lifespan was examined by transferring $^{111}$In-labeled platelets from donor mice to age-matched mice of the same strain.

‡$P < .001$ versus data in young (1 month) $F_1$ mice.

Dickinson) by gating to exclude debris. Unlabeled platelets were first analyzed to determine the highest level of autofluorescence, and then the percentage of positive platelets was calculated from the total platelet count above the level of autofluorescence.

*Platelet counts.* Blood (20 $\mu L$) was diluted in buffer containing ammonium oxalate (Unopette kits, Becton Dickinson). Platelets were counted using a hematocytometer under a phase-contrast microscope.

## RESULTS

Table 1 summarizes the platelet counts, PAA values, and platelet survivals of variously aged W/B $F_1$ mice. Male W/B $F_1$ mice gradually showed thrombocytopenia with age, and a significant decrease in platelet counts was observed in mice aged more than 4 months. In contrast, the parent mice (NZW and BXSB) and female W/B $F_1$ (3 months) mice showed normal platelet counts, as did normal BALB/c mice. PAAs were positive in more than 2-month-old W/B $F_1$ mice; percent-positive platelets increased with age (Table 1 and Fig 1). The mean platelet survival of BALB/c mice was 2.11 days. Male W/B $F_1$ mice more than 2 months of age showed significantly reduced PLS compared with male W/B $F_1$ mice at the age of 1 month. As shown in Fig 2, there were statistically significant correlations between the increase in percent-positive values of PAAs and either the reduction in PLS ($r = -.80$, $P < .01$) or the decrease in platelet counts ($r = -.69$, $P < .01$). There was also a statistically significant correlation between the reduction in PLS and the decrease in platelet counts ($r = .82$, $P < .01$). It should be noted that the reduction in PLS and the increase in PAA values were observed in male W/B $F_1$ mice even at the age of 2 months, although the platelet count was almost normal.

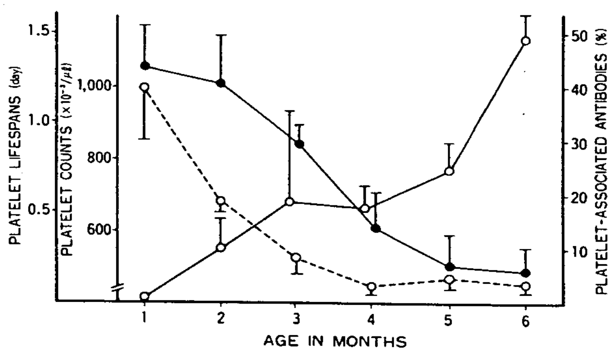


**Fig 1. Correlations between increased PAA values (O—O) and either reduced PLS (O---O) or decreased platelet counts (●—●) in male W/B $F_1$ mice. N values for the individual points are shown in Table 1.**

To elucidate whether the reduced PLS was due to the abnormality of the platelets themselves in male W/B $F_1$ mice or plasma factors, we performed platelet transfer experiments between male W/B $F_1$ mice and either female W/B $F_1$ or normal BALB/c mice.

As shown in Table 2, normal BALB/c (6 months) and female W/B $F_1$ (3 months) mouse platelets that had been transferred to male W/B $F_1$ (6 months) mice were immediately cleared from circulation after injection; PLS were 0.15 ± 0.28 days and 0.21 ± 0.04 days. Platelets transferred

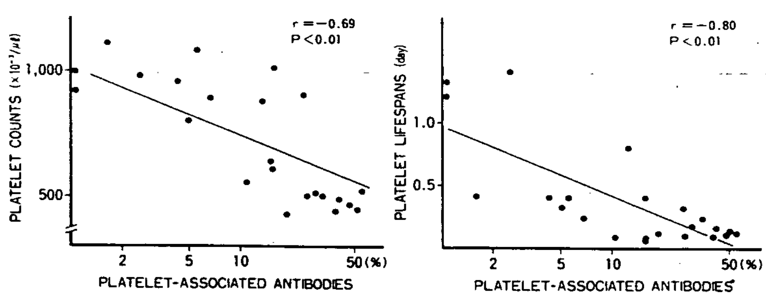


**Fig 2. Relationships between age and platelet counts, PAA values, and PLS in male W/B $F_1$ mice.**

**Table 2. Platelet Transfer Experiments Between Male W/B $F_1$ and Either Female W/B $F_1$ or Normal BALB/c Mice**

| Donor Mouse | | | | Recipient Mouse | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Mouse | Sex | Age (mo) | PAA (%) | Mouse | No. Examined | Sex | Age (mo) | PAA (%) | Platelet Survival (d) |
| BALB/c | M | 6 | 1.6 ± 1.0 | BALB/c | 6 | M | 6 | 1.6 ± 1.0 | 2.11 ± 0.28 |
| BALB/c | M | 6 | 1.7 | W/B $F_1$ | 4 | M | 6 | 44.6 ± 6.3 | 0.15 ± 0.03* |
| W/B $F_1$ | M | 6 | 41.2 | BALB/c | 4 | M | 6 | 1.6 ± 0.5 | 0.11 ± 0.02* |
| W/B $F_1$ | F | 3 | 1.9 | W/B $F_1$ | 4 | M | 6 | 47.0 ± 4.9 | 0.21 ± 0.04* |
| W/B $F_1$ | M | 6 | 39.0 | W/B $F_1$ | 4 | F | 3 | 3.0 ± 2.4 | 0.12 ± 0.04* |

*$P < .001$ versus data of BALB/c (6 months) platelet survival transferred to BALB/c mice (6 months).

from male W/B $F_1$ mice (6 months) to BALB/c mice (6 months) were also cleared from circulation immediately (0.11 ± 0.02 days). Platelets transferred from male W/B $F_1$ mice (6 months) to female W/B $F_1$ mice (3 months) were also cleared from circulation immediately (0.12 ± 0.04 days).

## DISCUSSION

In this study, we demonstrate that male W/B $F_1$ mice develop thrombocytopenia, increased anti-platelet antibodies on their platelet surface, and reduced platelet lifespans with age. There were statistically significant correlations between the increased PAA values and either the reduced PLS or the decreased platelet counts. From the present results and our previous findings of megakaryocytosis in the bone marrow,[5] we consider that the mechanism of thrombocytopenia in male W/B $F_1$ mice is due to increased PAAs resulting in the acceleration of platelet destruction, but not to decreased platelet production.

W/B $F_1$ mice are well-known for developing systemic lupus erythematosus (SLE)-like diseases and their expression of high levels of circulating immune complexes (CICs).[9-11] CIC-mediated thrombocytopenia was also demonstrated in patients with SLE.[12] Moreover, it is known that the CICs bind to specific glycoproteins in some drug-induced thrombocytopenia.[13] Although murine platelets have no Fc receptor, they possess C3b receptors capable of binding to CICs.[14,15] It is conceivable that CICs are involved in the reduced PLS in these mice. However, other typical SLE-prone mice, such as NZB, MRL/1pr, and BXSB, which show high CIC levels, have normal platelet counts.[16,17] Furthermore, the sera from BXSB and MRL/1pr mice do not bind to the platelets of BALB/c.[5] From these results, it seems unlikely that increased PAAs in W/B $F_1$ mice are due to the binding of CICs to the platelet surface. Therefore, we think that CICs do not play an important role in platelet destruction in W/B $F_1$ mice. In fact, CICs are often observed in patients with ITP, but it is known that they have little influence on platelet destruction.[18,19]

Significantly reduced PLS and increased PAA values were noted in male W/B $F_1$ mice even at the age of 2 months, although their platelet counts were almost normal. We speculate that platelet destruction was mild and compensated for by increased production of platelets in these mice of that age. Similar kinetic observations have also been reported in patients with ITP after splenectomy.[20]

The immediate clearance of female W/B $F_1$ and normal BALB/c platelets transferred to male W/B $F_1$ mice indicates the presence of circulating anti-platelet antibodies in the plasma of old male W/B $F_1$ mice, as we previously demonstrated.[5] Furthermore, old male W/B $F_1$ mouse platelets transferred to BALB/c mice were also cleared from circulation immediately. As shown in Fig 2, the significant correlations between the increase in PAA values and the decrease in PLS or platelet counts strongly suggest that the immediate clearance of transferred platelets is due to the presence of antibodies (PAAs and circulating anti-platelet antibodies), but not due to an abnormal host environment, such as hyperfunction of the reticuloendothelial system in old male W/B $F_1$ mice.

We have no data to show conclusively the presence of autoantibodies to platelets. We are not only in the process of verifying to which platelet epitopes antibodies are produced, but also clarifying where and how the platelets of W/B $F_1$ mice are destroyed.

Because the mechanism of thrombocytopenia observed here appears similar to that of human ITP, this animal could serve as a useful model for investigating the effectiveness and mode of action of new therapeutic agents in human ITP.

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